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(54) Title: BLOOD AFFINITY DIAGNOSTIC METHOD

(57) Abstract

Novel methods and devices where capillary action is the motive force for movement of an aqueous assay medium, where first and second specific binding pair members are bound to the surface. Complex formation with the first specific binding pair member inhibits complex formation with the second binding pair member. The distance traveled by the homologous specific binding pair member is indicative of the presence of an analyte. The homologous second binding pair member provides for a detectable signal. For determining blood cell type with binding to the red blood cell, antibodies to the red blood cell type antigen are employed and the formation of a relatively well defined front in a zone containing the antibody to the blood cell type antigen is indicative of the blood cell type.

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BLOOD AFFINITY DIAGNOSTIC METHOD

FIELD OF THE INVENTION

Diagnostic procedures and apparatus are provided for rapid determination of blood type and the presence of analytes in blood.

BACKGROUND OF THE INVENTION

The market for measuring a specific chemical entity in a sample has undergone extraordinary expansion. One of the major areas of interest has been the detection of endogeneous or exogeneous substances in physiological fluids, such as blood, serum, urine, saliva, and the like. For many purposes, there is an interest in detecting a particular chemical entity in blood. A large number of the tests require the removal of the red blood cells, because of the many interfering properties of the red blood cells. Thus, the serum must be separated from the red blood cells in order to make the determination.

There are many ways for removing red blood cells and providing serum. Centrifugation will suffice. Certain membranes have been reported as being useful for this purpose. However, separations introduce a possibility for error, frequently increase the time necessary for determination, and add extra costs for the determination. It is therefore of sufficient interest to be able to devise techniques which allow for the use of whole blood in an efficient and accurate manner.

BRIEF DESCRIPTION OF THE RELEVANT LITERATURE

A number of patents describe specific binding assays in biological fluids, especially serum, such as Reckel et al., U.S. Patent No. 4,595,654; Bishop, U.S. Patent No. 4,436,824; Wagner et al., U.S. Patent No. 4,205,058; and Prince et al., EPA 85/110742.5 discloses an assay for the simultaneous detection of an antigen and an antibody in serum. Zuk, U.S. Patent No. 4,435,504 employs a bibulous element containing specific binding members to react with the analyte, while Zuk, U.S. Patent No. 4,594,327, provides an assay where red blood cells are removed from an assay medium containing whole blood and the analyte determined.

SUMMARY OF THE INVENTION

Methods, compositions and devices are provided for detecting an analyte. The device employs first and second binding pair members ("SBPM") which are members of different specific binding pairs or for detection of a surface epitope as part of intact membrane, a specific binding pair receptor for the surface epitope. The SBPM's will be non-diffusively bound to a substrate capable of participating in capillary transport.

Where the first and second SBPM's are employed, they will be bound to the solid substrate of a device in a manner which inhibits simultaneous binding and complex formation with first and second SBPM's bound in close juxtaposition to the surface. The first SBPM will bind to an analyte of interest. The second SBPM will bind to a signal mediator which allows for detection of the signal mediator on the solid substrate.

The assay is performed by contacting the device with the sample suspected of containing the analyte and containing the signal mediator. The dis-

tance the signal mediator travels will be related to the amount of analyte in the sample. With the surface epitope, different SPBM's will be used along individual paths and the formation of a detectable front used as an indication of the presence of the surface epitope.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagrammatic view of an assay employing a strip; and

Fig. 2 is a diagrammatic view of an assay employing a packed capillary showing the capillary before the assay and after the assay.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods, compositions and devices are provided for detecting the presence of an analyte of interest. The devices provide for capillary transport of the assay medium. Bound to the device are first and second specific binding pair members ("SBPM's"), where the first and second binding pair members are members of different specific binding pairs. In a special case, where the analyte is a surface epitope and is detected as part of an intact membrane, particularly as part of an intact cell, a single SBPM may be employed which binds to the surface epitope.

The first SBPM will be specific for the analyte of interest. The second SBPM will be specific for a signal mediator. The signal mediator is a molecule, which provides for detection, either directly or indirectly, of the presence of the signal mediator at a point on the surface of the device. The signal mediator permits the determination of the path length which the signal mediator traverses. An exception is where a surface epitope is to be detected, where a single SBPM

is employed in as many individual paths or two SBPM's per path for the number of epitopes of interest.

The method employs a device which is characterized by providing for capillary transport of an aqueous medium. Non-diffusively substantially uniformly bound to a surface are first and second SBPM's of different binding pairs. The SBPM's bind to form a non-covalent complex, where the conformation and charge distribution of the surface of one of the pair members is complemented by the conformation and charge distribution of the other pair member to define an homologous pair. The homologous pairs form complexes, which for purposes of the subject assays are substantially irreversible during the time of the test. The SBPM's are capable of binding to each other and having binding affinities of an order of at least 10^2, usually at least 10^3, greater than other closely related compounds, generally having a Ka of at least about 10^8liters/mole.

The assay is initiated by contacting the device at a predetermined site with the sample. The sample migrates by virtue of capillary action carrying with it any analyte and the signal mediator. The rate of migration of the signal mediator will generally be not significantly faster than the analyte, preferably substantially slower. As the analyte and the signal mediator migrate along the path of flow, the analyte will bind to its homologous SBPM inhibiting the binding of the signal mediator to its homologous SBPM. Since the analyte will normally precede the signal mediator, as the signal mediator moves through areas where analyte has bound to its homologous pair member, the signal mediator will migrate through those areas and continue to migrate until the analyte has been substantially exhausted and both first and second SBPM's are available for complex formation. The signal mediator will now bind to its homologous pair member until it, in turn, is exhausted or some other mechanism oper-

ates to build up a front which inhibits further transport of the signal mediator. By determinating the path length of the signal mediator with the sample, as compared to the path length with a sample having a known amount of analyte, one can qualitatively, semi-quantitatively or quantitatively determine the presence and amount of the analyte in the sample.

Where the signal mediator is a large particle, such as a cell, it is found that the binding of the signal mediator to its homologous pair member results in formation of a barrier or dam to which additional cells accrete, providing for a fairly defined front. This front, then provides the measure of the path length of the signal mediator.

The specific binding pair member will be somewhat arbitrarily divided into ligands and receptors. A ligand may be any compound for which there is a reciprocal receptor. Thus, the two members of the specific binding pair are functionally defined in that one is able to distinguish the specific binding pair members from other binding pair members by their ability to form a strong non-covalent complex with each other. While the receptor is normally a macromolecule, the ligand may or may not be a macromolecule. For the most part ligands will be organic molecules of at least about 125 daltons (Da) and may be many millions of daltons.

The ligands may be individual molecules, complexes, aggregations, or the like. Ligands may include small molecules, such as drugs, antibiotics, hormones, sugars, oligonucleotides, neurotransmitters, oligopeptides, polymeric molecules or macromolecules, such as enzymes, surface epitopes, structural proteins, polysaccharides, nucleic acids, or aggregations of molecules, such as viruses, organelles, such as nuclei, plastids, lysosomes, and peroxisomes, cells, such as bacteria, pathogens, mammalian cells, neoplastic cells,

blood cells, such as lymphocytes, leukocytes, erythrocytes, and monocytes, or fragments or components thereof. The receptors will generally be macromolecules, such as enzymes, immunoglobulins, lectins, nucleic acids, naturally occurring receptors, such as growth factor receptors, synaptic receptors, thyroxine binding globulin, and the like.

The analyte may be any ligand or receptor and will be detected by the binding of an epitopic site or its equivalent to its homologous receptor.

The device will be characterized by providing for capillary movement of an aqueous medium, by allowing for non-diffusive binding of a SBPM to a surface, either by non-covalent forces or by virtue of functionalization of the surface and covalent linkage to the SBPM.

Various materials may be used for the manufacture of the device. Particularly, bibulous materials may be used, such as cellulosic paper, nitrocellulosic membranes, glass fiber filters, silica gel or other particulate glass, polystyrene, polyethylene or other plastic support, or other material which is hydrophilic or made so by appropriate modification. Capillaries may be used, which may be formed of any translucent or transparent material, such as glass, plastics, e.g. polystyrene, acrylate, polyethylene, or the like, where the capillary is hydrophilic or made so by coating or other modification, in accordance with conventional techniques.

The device may be a strip of paper or membrane, which may or may not have a supporting backing. Generally, the strip will be at least 1mm, more usually 2mm wide and generally not more than about 10mm wide, usually not more than about 6mm wide. The length will depend upon the desired resolution, the width, the amount of analyte, the density of the SBPM's, and the like. Usually, the length will be at least about 5mm,

more usually at least about 10mm, and will usually not exceed about 50mm, more usually not exceeding about 30mm. Of particular interest are membranes, such as nitrocellulose membranes, e.g. Schleicher and Schuell, catalog #AE100. Conveniently, protein may be bound non-covalently to the membrane.

Capillaries which are employed may have an empty lumen or packed lumen, where the particles employed for the packing will be hydrophilic and allow for capillary transport. The specific binding pair member(s) may be bound to the surface of the capillary, to the packing, or both. Various particles may be employed, of a variety of materials, such as agarose, sepharose, bioglas, latex, or the like.

The lumen of the capillary may be as small as 0.5mm, and will usually not exceed about 5mm, more usually not exceed 2mm. The length of the capillary is not critical, usually exceeding about 5mm, and not exceeding about 100mm, more usually not exceeding about 75mm, and preferably not exceeding about 50mm. The medium will be capable of being transported by capillary action in a horizontal plane or against gravity.

The signal mediator is characterized by being any composition which migrates at a rate not significantly greater than the rate of migration of the analyte. The signal mediator will provide a detectable signal, either directly or indirectly, so that the presence of the signal mediator specifically bound to the surface of the device may be detected.

Of particular interest, because of having broad applicability, being present in whole blood and permitting analysis of blood type and analytes in whole blood and providing for a relatively sharp demarcation for measurement, is the use of red blood cells. Red blood cells provide for a characteristic red color. Furthermore, it is found that as the red blood cells specifically bind to their homologous SBPM bound to the

device surface, other red blood cells accrete to form a barrier which provides for a relatively sharp line of demarcation. This line of demarcation can be used to measure the path length that the red blood cells migrated as an indication of the presence or absence of an analyte. Thus, whole blood can be used without separation of the red blood cells, where the red blood cells provide an advantage in acting as a signal mediator, rather than an interferant, which is generally removed because the RBC's result in a background value and prevent detection of a different signal mediator.

Besides red blood cells, numerous other compounds may be employed either by themselves or linked to other materials, such as macromolecules, e.g. proteins, particles, e.g. agarose, latex, etc., or the like. Compounds which may be used include a wide variety of strongly absorbing dyes or other absorbing compositions, such as ferritin, carbon black, tetrazolium salts, gentian violet, etc.; fluorescers such as fluorescein, rhodamine, dansyl, phycobiliproteins, lanthanide chelates, etc.; enzymes, such as peroxidase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, cholinesterase, etc.; enzyme co-factors such as FAD, NADH, etc.; particles, such as magnetic particles, particles to which any of the former signal mediators have been bound, colloidal metal particles, cells, etc.

The signal mediator may provide for a signal either directly or indirectly. Where the signal is provided directly, only a physical measurement is required to detect the presence of the signal mediator bound to the device surface. Where, however, the signal is provided for indirectly, a second step will usually be required for detection of the signal mediator. Particularly, epitopic sites will be present on the signal mediator for which receptors are available. The receptors will be capable of binding to the signal

mediator and will provide for the detectable signal. Thus, one of the previously described signal mediators which allows for direct detection will be bound to the receptor. Usually, the signal mediator will have a plurality of epitopic sites to which the receptor may bind, thus providing for significant amplification of the signal. This technique finds analogy in sandwich assays which have found extensive exemplification in the literature, particularly with the enzyme label.

The manner in which comcommitant binding of homologous first and second SBPM's is inhibited can be achieved in a variety of ways. Depending upon the nature of the analyte and/or its homologous binding member, various techniques may be employed. For example, the two SBPM's may be covalently linked, either directly or by a linking group so as to be in close spatial proximity. Alternatively, the two different SBPM's may be bound non-diffusively, usually covalently, to a surface, where the relative proportions in light of the size of the homologous binding members provides for the desired inhibition of concommitant binding. In some instances, it may be desirable to differentially activate the surface, so as to have two different linking groups, where the different specific binding pair members will selectively link to one or the other linking groups. For example, mercapto groups and amino groups may be employed, where the mercapto group will preferentially bind to an activated olefin, e.g. maleimide, while carboxy groups would preferentially bind to the amino groups. By employing appropriately bifunctional compounds bound to the surface, the linking of the two specific binding members to a single molecule may be achieved, e.g. a polyfunctional polymer.

The ratio of the first specific binding pair member to the second SBPM will vary widely, usually

from about 0.1-1:1, more usually from about 0.3-1:1. The optimum ratio can be determined empirically.

For determining a surface epitope, one will normally use from one to two SBPM's, either ligand or receptor, which will usually be nondiffusively bound to the surface. Conveniently, individual paths will be provided for the number of surface binding proteins of interest or epitopes of interest, where the different epitopes of the same protein are of interest. Thus, one can have a plurality of paths adjacent to one another on a strip, where each of the paths are contacted simultaneously with the sample. The presence of a line of demarcation would be indicative of a particular surface epitope. Where the line of demarcation is not capable of detection, either visually or by instrument, the device may be contacted with an agent which binds to the cells, so as to provide for the detection of the cells. The presence of a broad, relatively concentrated, cell containing area would be indicative of the presence of the surface epitope. A path may have SBPM's of two or more different SBPM pairs, where each SBPM has an individual zone at successive distances along the path. So long as each cell has only one SBPM, the zone in which the cell is captured will determine which SBPM of interest is present on the membrane.

The sample may be any type of sample, which may be presented to the device as an aqueous liquid. Thus, the sample may be a solid, liquid or gas, may be a physiological fluid, such as blood, urine, plasma, serum, cerebrospinal fluid, ocular lens fluid, cytosol, or the like, water sample, soil sample, chemical processing sample, etc. The sample may be used directly or be subjected to various treatments, such as extraction, separation, dissolution, heating, cooling, chromatography, electrophoresis, or the like. The sample may be buffered with any of a variety of buffers, such

as bicarbonate, Tris, phosphate, barbital, MOPS, glycine, etc. Generally, the buffer will be present in from about 50mM to 0.5M. Other additives may also be included, such as enzyme inhibitors, bacteriostats, anticoagulants, proteins to inhibit nonspecific binding or other conventional additives.

Depending upon the nature of the analyte, various reagents may be added in order to perform the assay. For example, where the analyte is a ligand, excess receptor may be added, so as to completely bind any ligand present in the assay medium. Also bound to the device will be ligand, which will bind excess receptor. Thus, the excess receptor will serve to inhibit the simultaneous binding of the second SBPM partner. Similarly, where a receptor is involved, one can do the opposite and provide for the ligand in excess, where the excess ligand will bind to the receptor on the device surface. In this instance, the distance the signal mediator travels will be inversely related to the amount of analyte in the sample.

Where a signal mediator is not present in the sample, a signal mediator may be added. The signal mediator may be a small organic molecule of from about 150-2000Da, an intermediate sized molecule of from about 2000 to about 1,000,000Da or a larger molecule or other composition, such as molecular aggregations, such as hemoglobin, polysubunit proteins, organelles, cells, particles, such as metal particles, magnetic particles, fluorescent particles, and the like.

The signal may be as a result of light absorption, fluorescence, electromagnetic force, e.g. magnetic, enzymes, which provide for a light-absorbing product, particularly in the ultraviolet and visible range, a fluorescent product or other detectable product, enzyme co-factors, which can be detected with enzymes, and the like.

The signal mediator allows for detection of the molecule and will be inhibited from binding to its reciprocal binding member in the presence of complex formation of the first specific binding pair. Particulate signal mediators will be relatively large, usually at least about 10nm, more usually about 50nm and may be 50μm or larger, preferably being in the range of 1μm to 20μm.

Where the signal mediator does not naturally allow for a ready means of detection, the signal mediator may be modified to provide for such detection or may provide a basis for non-covalent binding of molecules which allow for detection. A signal mediator which naturally permits detection is a red blood cell, ferretin, hemoglobin, or the like. Similarly, particles, such as metal particles and colloids, carbon black or the like may be employed where the particles may be conjugated to a specific binding pair member. Colored particles or fluorescent particles may be employed where latex particles, agarose particles, sepharose particles, or the like may be colored by conjugation to dyes, may be made fluorescent to conjugation to fluorescers and may be conjugated to the specific binding pair for binding to the reciprocal specific binding pair member.

The assay medium will normally be at a pH in the range of about 4 to 10, more usually 6 to 9, so as to provide for enhanced complex formation between reciprocal binding members. Other solvents may also be included, particularly polar organic solvents, such as alkanols, ethers, amides, or the like, which serve to reduce the viscosity of the assay medium, change the binding characteristics of the SBPM's, or provide some other desirable characteristic.

After the sample has undergone treatment, if appropriate, the device may then be contacted with the sample. The contact may be at an edge, at an end, at a

position somewhere near the end, where the device may be dipped into the sample, touched at the surface of the sample, a small amount of the sample applied to the device, or other convenient means for bringing the device and sample into contact. In many situations it may be sufficient to allow for ambient conditions. In other situations, temperature control may be required, where the temperature may vary from about 0°C to 40°C. The time for the assay will usually be at least one second to one hour or more, usually varying from about 5sec to 30min.

After sufficient time for the assay medium to traverse the desired path distance, the distance traveled by the signal mediator may be determined. Where the signal mediator inherently permits determination, it may be directly determined either visually or by an instrument. Various spectrophotometers, fluorometers, magnetometers, reflectometers, etc., may be employed. However, where the signal mediator does not allow for a direct determination, further steps may be required, such as adding a conjugate of a molecule which allows for detection joined to a molecule which specifically binds to the signal mediator. In this instance, the device may be coated, sprayed, or otherwise contacted over the assay medium path with the conjugate which provides for the signal. In some instances, it will be desirable to wash away any nonspecific binding conjugate before determining the distance traversed by the signal mediator.

Where an enzyme is the label the substrate may be provided in a zone downstream from the region of the SBPMs. Thus, the SBPM region can be designed that a predetermined level of analyte in the sample will result in migration of the enzyme label into the substrate zone. A change of color in the substrate zone would indicate that the amount of analyte is above a predetermined level. If one wished to quantitate the

amount of analyte, anti-enzyme could be uniformly bound in the substrate zone, so that the enzyme label would migrate through the substrate until exhausted. By having a colored enzyme product which strongly binds to the surface, the colored border would indicate the amount of analyte present.

In order to quantitate the amount of analyte, one or more standards may be employed, where the assay is carried out with a known amount of analyte in a sample medium. In fact, devices may be provided which allow for the simultaneous performance of a standard and the sample for direct comparison. By comparing the observed result with the result obtained with a sample having a known amount of the analyte, one can relate distance quantitatively to the amount of analyte present in the sample.

Where one is interested in a surface epitope, one will be primarily concerned with the formation of a thick margin or front of cells, where the absence of such thick front will be indicative of the absence of the surface epitope. Thus, one need not be as concerned about the distance, but rather the formation of the dam or barrier cells at a site somewhat removed from the site of contact with the sample.

As a matter of convenience, kits can be provided of the device in conjunction with other reagents, such as the signal mediator, the signal mediator in conjunction with the detectable conjugate, standards, and other reagents, such as buffer, stabilizers, or the like. The amount of reagents will be related to the device to provide substantially optimum sensitivity for the assay.

The Figures indicate diagrammatically two different devices. The strip device 10 of Fig. 1 indicates the appearance of the device after performance of the assay. The device 10 has a bibulous base 12 to which are bound antibodies to RBC 14 and antigen 16.

The assay is for the presence of a receptor 18 for the antigen 16, for example, an antibody to the antigen. Blood is applied at one end 20 of the base and migrates along the base. In the case of a negative result, the red blood cells 22 migrate along the base and are captured by the anti-RBC's 14. They mass and result in a barrier which prevents their further migration. In the case of the positive result, the anti-antigen 18 travels faster than the RBC's and binds to the antigen 16. The presence of the anti-antigen 18 inhibits the binding of the RBC's to the anti-RBC's 14. Once the anti-antigen 18 is exhausted, the anti-RBC's 14 may then bind to the RBC's 22. In the embodiment depicted, the zone in which the anti-RBC's 14 and the antigens 16 are present becomes saturated with anti-antigen 18, so that the RBC's continue traveling further up the base and create a red zone 24.

In Fig. 2 a capillary device 30 is depicted. At the beginning of the assay, the capillary device 30 is dipped into sample 32. The capillary 34 is packed with particles 36 to which are bound antibody conjugates 38. The antibody conjugates 38 are also bound to the wall of capillary 34. The antibody conjugate comprises an antibody indicated by Y and a hapten indicated by a black dot. The device 30 is removed from the sample 32 after a sufficient amount of sample has entered the capillary 34 or the device 30 may remain in the sample until sample 32 has migrated to the top of the capillary 34.

In Fig. 2b the assay is completed. The assay is for hapten, where a known amount of excess antibody to hapten, anti-hapten has been added to bond to any hapten present in the sample 32. Anti-hapten 40 which has not bound to hapten in the sample 32 may bind to hapten of the conjugate 38. Binding of anti-hapten 40 to the conjugate 38 prevents cells 42 from binding to the conjugate, so that the cells migrate past the zone

where the anti-hapten 40 is bound to the conjugate 38. Once the anti-hapten 40 is exhausted, the cells 42 bind to the conjugate 38 and additional cells begin to non-specifically bind to form a front 44. The distance of the front from the end of the capillary 34 is thus a measure of the amount of the hapten in the sample. The shorter the distance, the greater amount of analyte in the sample.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

METHODS

1. Preparation of Salivary Glycoprotein Fractions

In order to determine the presence of an anti-A or anti-B circulating antibody in a patient, one must have the specific antigen available for detection. This is accomplished by isolating soluble glycoproteins from the saliva of individuals of Type A or Type B, ABO blood groups. These glycoproteins are then utilized as the second specific binding pair on the affinity chromatograph. The soluble glycoproteins were isolated from individuals known to be secretors of Type A or Type B glycoproteins on the basis of a standard saliva hemagglutination inhibition test. Following the procedure of Barrie et al. (J. Immunogenetics 10: 41-44, 1983), saliva was heated in a boiling water bath for 15 min. immediately after collection. The cooled saliva was centrifuged for 10min at 2000rpm in an IEC Centra-7 centrifuge to sediment particulate material and the supernatant fraction was mixed with six volumes of 95% ethanol. The mixtures were stored overnight at 4°C and centrifuged 10min at 2000rpm to sediment the antigenic glycoprotein fraction. This glycoprotein pellet was washed once with 95% ethanol, sedimented at 2000rpm, and dried under vacuum. The final pellets were resuspended in saline for immobilization on chromatography strips as described below.

2. Biotinylation of Anti-Red Blood Cell Immunoglobulin

Avidin is a protein with a strong binding affinity for biotin and therefore was used in combination with anti-red blood cell immunoglobulin to demonstrate the ability to detect the presence of avidin in a blood serum sample. Three ml of an IgG fraction of rabbit anti-serum directed against human red blood cells

(Cooper Biomedical) was reconstituted to 30mg protein/ml and dialyzed overnight at 4°C against 1 L of 0.1 molar sodium phosphate buffer, pH 7.6. Aliquots of this dialyzed IgG were reacted for 2 hr at 23°C with N-hydroxysuccinimidobiotin (NHS-biotin) with molar ratios: (IgG: NHS-biotin) between 1:1 and 1:100. The aliquots were dialyzed against two changes of 1 L of phosphate buffered saline (PBS; 0.9% NaCl buffered with 20mM sodium phosphate, pH 7.2) prior to immobilization of the biotin-immunoglobulin conjugates.

3. Immobilization of Proteins on Cellulose Supports

A variety of reagents exist for covalent attachment of proteins to these materials, including cyanogen bromide, cyanuric chloride, and carbonyldiimidazole. An example of one such method, adapted from published procedures (Bethell, et al., J. Chromatography 219: 361-371, 1981; Zuk, et al., Clinical Chemistry 31: 1144-1150, 1985), is described below for the immobilization of biotin-immunoglobulin conjugates on Whatman 31 ET Chrom paper.

Four sheets of Whatman 31 ET Chrom paper were cut into 4cm x 17cm strips with the long axis parallel to the machine direction of the paper. These sheets were immersed in 136ml of 0.2M 1,1'-carbonyldiimidazole (CDI) in anhydrous dichloromethane in a flat bottom glass vessel and reacted for 2 hr at 23° with rotary agitation. The paper was washed with three changes of dichloromethane (150ml each for 5 min), dried under nitrogen, and cut perpendicular to the long axis into 3mm x 40mm strips.

Proteins were added to this activated paper by contacting a 5µl sample of the protein conjugate solution dissolved in PBS (1 to 30mg/ml) onto a local region of the CDI-activated strips, 10mm from one end, which was designated as the bottom of the strip. These strips with the conjugate solution were incubated for

1hr at 23°C in an atmosphere saturated with water, washed with agitation for 10 min in excess PBS, and dried in a convection oven for 10 min at 37°C. By this procedure, the proteins were immobilized in a 4 to 5mm zone or region centered 10mm from the bottom of the strip. Alternatively, the strips were wetted uniformly with 50µl of the protein conjugate solution and then incubated, washed, and dried as described. These strips were then used for forward blood typing or for the detection of analytes.

Strips for reverse blood typing where the presence of anti-B antibodies in a Type A individual, or anti-A antibodies in a Type B individual were prepared by the immobilization of mixtures of anti-red blood cell immunoglobulin and the salivary glycoprotein antigen, in the same manner as previously described for the anti-red blood cell conjugates. Similarly, strips for the detection of serum immunoglobulins against HTLV-III were produced through the use of antibody mixtures of anti-red blood cell immunoglobulin and HTLV-III inactivated virus.

4. Preparation of Strips for Forward Blood Typing

Forward blood typing refers to the binding of the red blood cell with the immunoglobins specific for blood group antigens on their surface, thereby creating a barrier to further movement of the red blood cell in the affinity chromatograph. Strips were cut from 31 ET Chrom paper as described above with the ommission of the CDI activation step. Five microliters of anti-A or anti-B human serum immunoglobulin (American Dade; Hemagglutination titer = 1/1024) was applied 1cm from the bottom of each strip and allowed to dry in the air. This immunoglobulin was not covalently bound and was merely dried into the paper strips. When the red blood cell containing sample was applied, the immunoglobulins partially dissolved and bound to the red blood cells.

5. General Test Procedure with Paper Strips

Paper strips were dipped vertically into blood samples to a depth of 2mm and the blood was permitted to ascend within the strips for 5 to 10min. The strips were then removed from the blood and air dried. The distance of the red cell front from the bottom of the strips was measured for quantitation of the results. Following addition of the red blood cell containing sample, anti-blood group immunoglobulins, which had been dried within the strips were solubilized as the blood ascended, thereby promoting agglutination of the red blood cells. In "forward" blood typing, strips scored as negative, if the red blood cells traveled further than blood known to be negative for the complementary immunoglobulin-known type O blood, which has no A or B surface antigens, and serves as a control in forward blood typing tests.

In a reverse typing assay, using glycoprotein "A" antigen, strips were scored as positive if the red blood cells migrated further than the strips developed in blood known to be negative for the complementary immunoglobulin. If anti-A antibody was present as an analyte in the sample, it bound to the glycoprotein, thereby blocking binding of the red blood cells to the red blood cell receptors and allowing transport (Example IV). Blood known to be type AB, which contains no anti-A or -B, may serve as a control for reverse blood typing. Similarly if antibody to the HTLV-III virus was present, the antibody bound to the HTLV-III antigen and permitted movement of the red blood cells further into the affinity chromatograph (Example II). Strips containing HTLV-III antigen and red blood cell immunoglobins were scored as positive if the red blood cells migrated farther than strips used to test samples known to be negative for the HTLV-III antibody.

Paper strips containing immobilized biotinylated anti-red blood cell immunoglobulins permitted detection of avidin. The movement of red blood cells within the strips was proportional to the concentration of avidin added to the blood samples. Therefore, the biotin covalently attached to the immunoglobulin with binding specificity for the red blood cell, and allowed the binding of avidin to hinder the ability of that immunoglobulin to bind to the red blood cell (Example III). Scoring was the same as that used for detection of anti-A or HTLV-III antibodies in sample.

EXAMPLE I

Forward Testing of ABO Blood Group

A forward test of the ABO blood group of a blood sample was conducted in the following manner.

Test strips (3mm x 40mm) as previously described, had 5µl of anti-A antibody attached to the CDI activated paper 10mm from the bottom end. After washing and drying, each strip was dipped into a 10ml beaker containing 1ml blood (20 drops whole blood) and allowed to sit at room temperature for 10min. Assays were run in duplicate with the results tabulated in Table 1:

Table 1

Blood Type	Ab*Type	Distance RBC Traveled (in mm)
A	anti-A	14, 16
B	anti-A	21, 23
A	anti-A	14, 14
B	anti-A	20, 17

*Ab - antibody immobilized within the test strip

When 5µl of antibody was dried on unactivated paper without washing, the results were as in Table 2:

Table 2

Blood Type	Ab*Type	Distance RBC Traveled (in min)
A	anti-A	9
B	anti-A	19

*Ab-antibody dried into test strip

EXAMPLE II

Determining the Presence of HTLV-III (AIDS) Antibody

The detection of HTLV-III antibody as an analyte in a whole blood sample was performed in the following manner.

The HTLV-III antigen was prepared in the following manner: Tissue culture cells of the H9HXB strain were infected with HTLV-III, then pelleted and lysed with Triton X-100. Coupling was to CDI activated paper (3mm x 40mm) with 5µl of anti-RBC antibody and 5µl of HTLV-III antigen simultaneously bound as previously described. Packed type O red blood cells (1ml) were mixed with human serum (1ml) from either an anti-HTLV-III positive (P) or negative (N) donor. Table 3 indicates the results:

Table 3

HTLV-III Donor Serum & RBC Type		Antibody Type and Antigen	Distance RBC Traveled (in mm)
Negative	O	Anti-RBC HTLVIII	14, 16
Positive	O	Anti-RBC HTLVIII	18, 17.5

EXAMPLE III

Detection of Avidin by Biotin Conjugated Antibody

The detection of avidin, a dissolved protein, was accomplished by using a biotin conjugated antibody specific for red blood cells.

Biotin was covalently bound to anti-RBC antibody as previously described. The molar ratios of antibody and NHS Biotin were 1:1, 1:10 and 1:100. The 1:100 ratio probably caused significant change in the antibody structure. A 5µl sample was placed 10mm from the end of CDI activated paper strips (3mm x 40mm) and prepared as previously described. A mixture of blood type A and avidin to final concentration of 1mg/ml was prepared. The results are indicated below:

	Distance RBC Traveled (mm)		
Antibody: Biotin	1:1	1:10	1:100
RBC with avidin	14	16	15
RBC without avidin	12	13	16

The presence of the avidin analyte promotes movement, except at high biotin concentrations where the antibody is compromised.

EXAMPLE IV

Detection of Serum Antibodies Specific for Anti-A or Anti-B by the use of Salivary Glycoproteins

The presence of anti-A or anti-B antibody in blood serum is a measure of the likelihood of strong reaction against added blood of that type. Anti-A antibody is detected by the salivary glycoprotein containing the A-antigen and anti-B antibody in the blood serum is detected by utilizing salivary glycoprotein B.

The ABO blood type of an individual may be determined by detecting the presence of anti-A antibody

in a type-B person; anti-B in type-A, and both anti-A and anti-B in type-O. This serum antibody may be detected using salivary glycoprotein as antigens since they contain the same antigenic specificity as antigens on the surface of red blood cells. Salivary glycoprotein was prepared as previously described, with a hemagglutination inhibition titer of 1/128. Anti-RBC antibody (1ml) plus 1ml glycoprotein A were mixed. In one experiment 50µl of the mixture was used to saturate a CDI activated paper strip (3 x 40mm). In a second experiment 5µl of the mixture was placed 10mm from the bottom. The strips were placed into the blood sample for 5min at room temperature. The results with 50µl saturated strips is:

Blood Type	Distance RBC Travelled
A	9.0 m
B	11.5 mm

The type-B blood contained anti-A antibody which bound to the type-A glycoprotein and allowed the RBC to move further into the affinity chromatograph.

When 5µl samples of the anti-RBC antibody-glycoprotein A mixture were spotted onto the chromatography strip the results were:

Blood Type	Distance RBC Travelled
A	18.5, 18.5
B	21.0, 22.5

Therefore, the type-B blood demonstrably contained anti-A antibody, as expected.

EXAMPLE V

Biotinylated Anti-RBC Antibody Determination of Avidin

In the next study Schleicher and Schuell #AE100 12µm pore size nitrocellulose was taped onto a plastic backing with double sided tape (3M #666) and cut into 3mm x 40mm strips.

One µl of a 30mg/ml of biotinylated anti-RBC antibody is spotted 10mm from the bottom and the strips incubated for 5min at room temperature at 100% humidity. The strips are then placed in 100mm Petri dishes and washed in 20ml PBS, 0.1% polyvinylpyrrolidone (~10kDA) at room temperature for 30min with rotary agitation (50Hz). The strips are air-dried for 5min in a convection oven at 37°C.

The assays are performed as follows: Into a 1.5ml microcentrifuge tube is added 20µl of a blood sample and the strip is immersed in the blood sample. The strips are incubated at room temperature for 10 min removed from the tube and air-dried.

In the above assay protocol, RBC's in whole blood migrated 10mm, while in whole blood with 1mg avidin/ml, the RBC's traveled 15 and 16mm in two determinations. In a study where the avidin concentration was varied, at 1mg avidin/ml the RBC migration distance was 18mm, while at 0.5mg avidin/ml the distance was 11.5mm.

EXAMPLE VI

Capillary Determination of Blood Types

The use of capillaries was exemplified by filling Drummond Microcaps, 0.1µl, 89.2µm i.d. with 12mg/ml human monoclonal antibody against blood group substance A. After expelling the liquid, the capillaries were air-dried. The capillaries were dipped in either type A or B blood for 5sec and the distance tra-

versed by the RBC's was measured. For type A blood in duplicate determinations, the distances were 7 and 5mm, while for type B blood, the distances were 15 and 16mm.

It is evident from the above results, that the subject method provides a rapid, accurate and convenient method for detecting a wide variety of analytes, particularly employing non-technical personel. In most instances, little skill is required and, as desired, readings can be made visually. There is, therefore, a broad spectrum of applications for the subject invention, in hospitals, laboratories, in the field, in doctor's offices, and the like. The invention finds particular application employing red blood cells, as an advantage in being able to use the red blood cells, as the indicator of the result. In this manner, when blood is being employed or other sample to which red blood cells may be added, the red blood cells act to accurately define the amount of analyte in the sample, rather than requiring that the red blood cells be removed as in other assays where they obscure the desired result. The subject invention is highly flexible in allowing for a wide variety of protocols depending on the nature of the analyte, the quantitation desired, or the like.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for detecting an analyte in an aqueous assay medium employing a device comprising first and second specific binding pair members, each of said first and second specific binding pair members being members of different specific binding pairs and non-diffusively bound to a surface in relative spatial relationship to inhibit the concommitant binding of the homologous members of said first and second specific binding pairs, said device providing for capillary transport of said assay medium along a predetermined path,

wherein said assay medium comprises a signal mediator, which is the homologous second specific binding pair member and provides a detectable signal when present as a member of a complex with said second specific binding pair member, and said first specific binding pair member is cross-reactive with said analyte or the homologous specific binding pair member, said method comprising:

contacting said sample with said device at one end of said path whereby said sample is transported by said capillary action along said path and said analyte or its homologous specific binding pair member travels at least about as fast as said signal mediator, whereby said signal mediator is inhibited from binding to its homologous binding pair member adjacent to complex formation of said first specific binding pair; and

determining the distance traversed by said signal mediator in comparison to a sample having a known amount of analyte.

2. A method according to Claim 1, wherein said device is a capillary.

3. A method according to Claim 1 wherein said device is a bibulous strip.

4. A method according to Claim 1, wherein said signal mediator is a particle.

5. A method according to Claim 1, wherein said particle is an erythrocyte.

6. A method according to Claim 5, wherein said sample is whole blood.

7. A method according to Claim 1, wherein said first and second binding pair members are covalently conjugated to each other.

8. A method according to Claim 1, wherein said second binding pair member is an immunoglobulin.

9. A method for detecting an analyte in an aqueous assay medium employing a bibulous strip device comprising first and second specific binding pair members, each of said first and second specific binding pair members being members of different specific binding pairs and non-diffusively bound to a surface in relative spatial relationship to inhibit the concommitant binding of the homologous members of said first and second specific binding pairs, said device providing for capillary transport of said assay medium along a predetermined path,

wherein said assay medium comprises a signal mediator, which is the homologous second specific binding pair member and provides a detectable signal when present as a member of a complex with said second specific binding pair member, and said first specific binding pair member is cross-reactive with said analyte

or the first homologous specific binding pair member, said method comprising:

contacting said sample with said device at one end of said path whereby said sample is transported by said capillary action along said path and said analyte or its homologous specific binding pair member travels faster than said signal mediator, whereby said signal mediator is inhibited from binding to its homologous binding pair member adjacent to complex formation of said first specific binding pair; and

determining the distance traversed by said signal mediator in comparison to a sample having a known amount of analyte.

10. A method according to Claim 9, wherein said bibulous strip is a cellulosic material.

11. A method according to Claim 10, wherein said cellulosic material is a nitrocellulose membrane.

12. A method according to Claim 9, wherein said first and second specific binding pair members are covalently conjugated to each other.

13. A method according to Claim 9, wherein said first and second specific binding pair members are independently bound to said bibulous strip.

14. A method according to Claim 9, wherein said analyte is a hapten, said first specific binding pair member is a hapten cross-reactive with said analyte; and

including the additional step of adding the homologous first specific binding pair member to said sample, prior to said contacting.

15. A method according to Claim 14, wherein said first specific binding pair member is covalently conjugated to said second specific binding pair member, wherein said second specific binding pair member is an antibody and said homologous second specific binding pair member is a particle.

16. A method according to Claim 15, wherein said particle is a red blood cell.

17. A method according to Claim 9, wherein said analyte is an antigen.

18. A method for detecting an analyte in an aqueous assay medium employing a capillary device comprising first and second specific binding pair members, each of said first and second specific binding pair members, being members of different specific binding pairs and bound to a surface of a capillary in relative spatial relationship to inhibit the concommitant binding of the homologous members of said first and second specific binding pairs, said device providing for capillary transport of said assay medium along a predetermined path,

wherein said assay medium comprises a signal mediator, which is the homologous second specific binding pair member and provides a detectable signal when present as a member of a complex with said second specific binding pair member, and said first specific binding pair member is cross-reactive with said analyte or the first homologous specific binding pair member, said method comprising:

contacting said sample with said device at one end of said path whereby said sample is transported by said capillary action along said path and said analyte or its homologous specific binding pair member travels at least about as fast as said signal mediator, whereby

said signal mediator is inhibited from binding to its homologous binding pair member adjacent to complex formation of said first specific binding pair; and

determining the distance traversed by said signal mediator in comparison to a sample having a known amount of analyte.

19. A method according to Claim 18, wherein said first and second specific binding pair members are covalently conjugated to each other.

20. A method according to Claim 18, wherein said first and second specific binding pair members are independently bound to said bibulous strip.

21. A method according to Claim 18, wherein said analyte is a hapten, said first specific binding pair member is a hapten cross-reactive with said analyte; and

including the additional step of adding the homologous first specific binding pair member to said sample, prior to said contacting.

22. A method according to Claim 21, wherein said first specific binding pair member is covalently conjugated to said second specific binding pair member, wherein said second specific binding pair member is an antibody and said homologous second specific binding pair member is a particle.

23. A method according to Claim 22, wherein said particle is a red blood cell.

24. A method according to Claim 18, wherein said analyte is an antigen.

25. A method for detecting blood type by reverse typing in a blood sample by detecting an antibody to a blood type antigen, employing a device comprising first and second specific binding pair members, said first specific binding pair member being an antigen cross-reactive with a blood type antigen, and a second specific binding pair member being specific for a red blood cell epitope other than a blood type antigen, said first and second specific binding pair members being bound to a surface in relative spatial relationship to inhibit the concommitant binding of said antibody and said red blood cell, said device providing for capillary transport of said blood sample along a predetermined path, said method comprising:

contacting said sample with said device at one end of said path whereby said sample is transported by said capillary action along said path and said red blood cells are inhibited from binding to said second specific binding pair member in the presence of binding of said antibody to said first specific binding pair member; and

determining the distance traversed by said red blood cells in comparison to a sample of known blood type.

26. A method for determining the presence of an epitopic side on a cell membrane in an aqueous assay medium employing a device comprising a specific binding pair member bound to a surface in sufficient amount to result in accumulation of said membrane at a relatively defined boundary upon binding of said epitopic site to said specific binding pair member, said device providing for capillary transport of said assay medium along a predetermined path,

wherein said cell membrane provides for a detectable signal or after the sample has traversed a predetermined path, the device is contacted with a

reagent specific for said membrane and capable of providing a detectable signal, said method comprising:

contacting said sample with said device at one end of said path whereby said sample is transported by said capillary action along said path; and

determining the distance traversed by said membrane by detecting said boundary.

27. A method according to Claim 26, wherein said device has at least two successive zones along said path, each of said zones having specific binding pair members for different epitopes present on a cell membrane.

28. A method according to Claim 26, wherein said membrane is an intact red blood cell membrane.

29. A device for the determination of an analyte, said device comprising:

a surface capable of providing capillary transport for an aqueous medium;

bound to said surface first and second specific binding pair members, where said first and second specific binding pair members are members of different specific binding pairs and are non-diffusively bound to said surface in spatial juxtaposition so that complex formation of said first specific binding pair inhibits complex formation of said second specific binding pair.

30. A device according to Claim 29, wherein said surface is a bibulous surface.

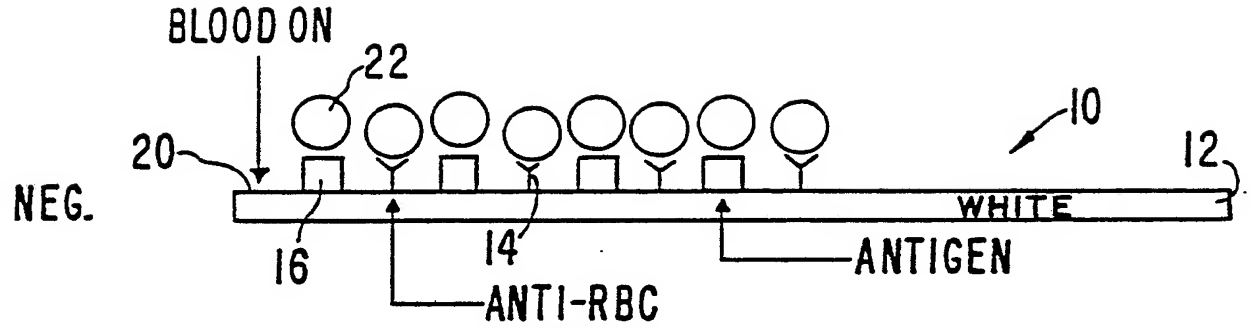
31. A device according to Claim 30, wherein said bibulous surface is a cellulosic surface.

32. A device according to Claim 31, wherein said cellulosic surface is a nitrocellulose membrane.

33. A device according to Claim 29, wherein said surface is a capillary surface.

34. A device according to Claim 29, wherein said second specific binding pair member binds to a red blood cell.

35. A device according to Claim 29, wherein said first specific binding pair member is a hapten.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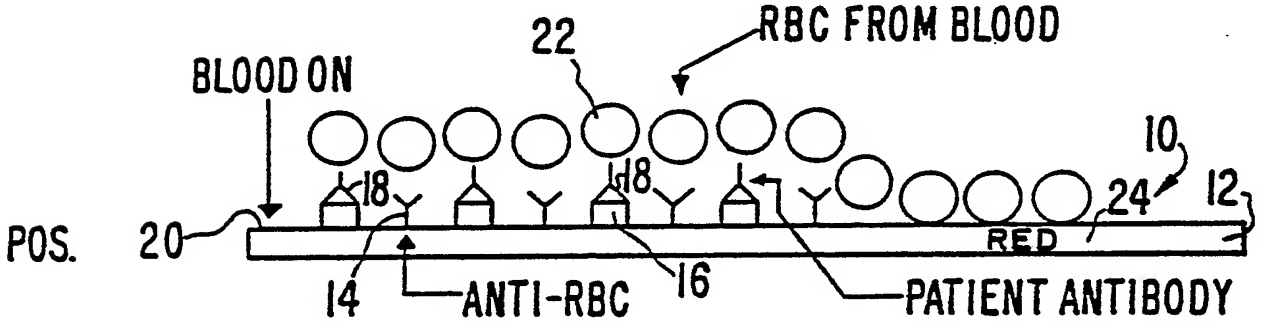
22
10
20
12
NEG.
WHITE
16
14
ANTIGEN
ANTI-RBC

FIG. 1a


22
RBC FROM BLOOD
BLOOD ON
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18
18
24
12
POS.
20
RED
14
ANTI-RBC
16
PATIENT ANTIBODY

FIG. 1b

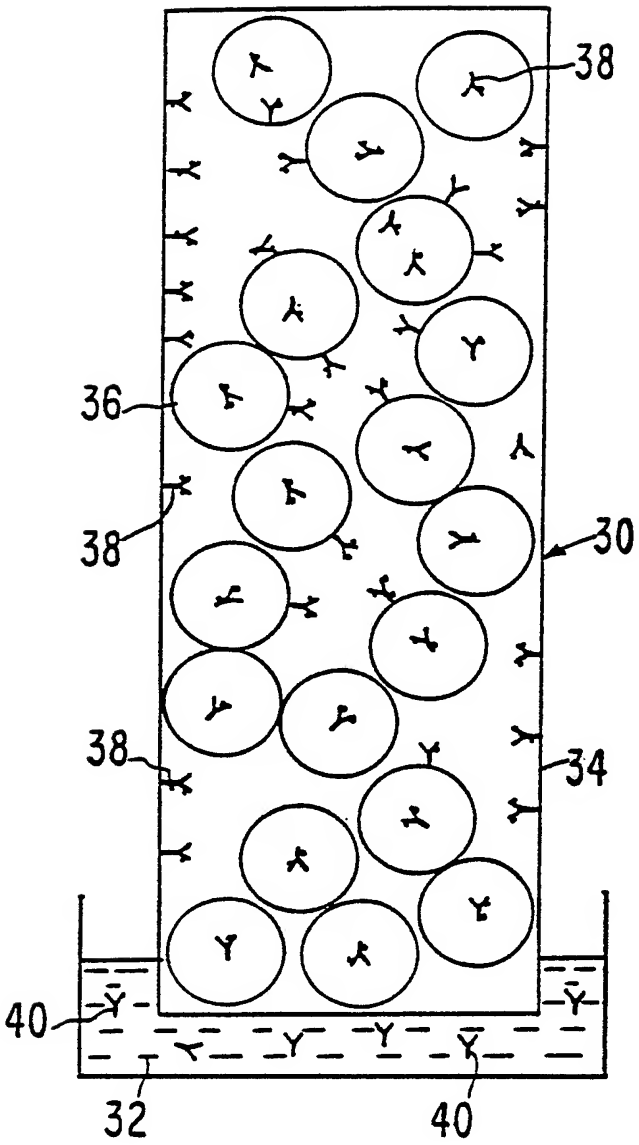

FIG_2a

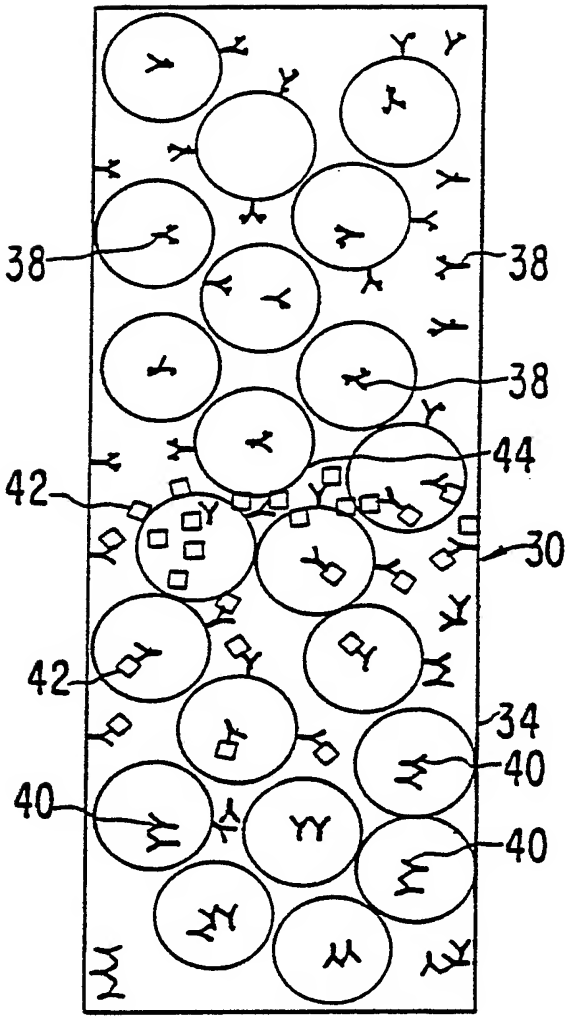

FIG_2b

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/02974

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): G01N 33/566; G01N 33/558, G01N 33/543, G01N 33/555
U.S.Cl.: 436/501, 514, 518, 520

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System	Classification Symbols
U.S.Cl.	436/501,514,515,518,519,520,530,535,541,807

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 5

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
Y	WO, A 8,603,839 (CERNY) 03 July 1986, see entire document.	1-35
Y	EP, A, 0,046,004 (SYVA) 17 February 1982, see entire document.	1-35
A	US, A, 4,517,288 (GIEGEL, ET AL) 14 May 1985, see entire document.	1-35
A	US, A, 4,594,327 (ZUK) 10 June 1986, see entire document.	1-35

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
08 FEBRUARY 1988	24 FEB 1988
International Searching Authority 1	**Signature of Authorized Officer 20**
ISA/US	Stephen C. Wieder Stephen C. Wieder